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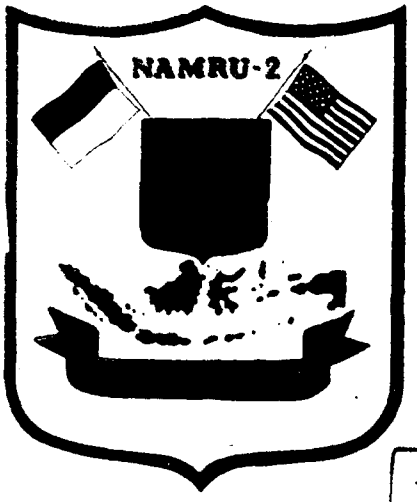

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EXPERIMENTAL VERTICAL TRANSMISSION OF JAPANESE ENCEPHALITIS VIRUS BY *CULEX TRITAENIORHYNCHUS* AND OTHER MOSQUITOES

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Abstract. Vertical transmission of Japanese encephalitis virus to the F1 adult stage was demonstrated in *Culex tritaeniorhynchus*, *Cx. annulus*, *Cx. quinquefasciatus*, and *Armigeres subalbatus*. Transmission to the F1 larval stage was demonstrated in *Cx. pipiens*, *Aedes vexans*, *Ae. alcasidi*, and *A. flavus*. In *Cx. tritaeniorhynchus*, vertical transmission rates (the percentage of parent females transmitting to progeny) varied (12–100%). Filial infection rates (the percentage of progeny infected) for a given mosquito virus combination were markedly affected by the interval of time between parental infection and oviposition, suggesting that vertical infection was not transovarial in nature but occurred at oviposition. Filial infection rates for *Cx. tritaeniorhynchus* also varied widely by family and, as measured in F1 larvae, rates in excess of 20% were observed in a family. Filial infection rates in *Cx. tritaeniorhynchus* F1 adults were about 4 times lower than those in larvae. Japanese encephalitis virus was sexually transmitted from male to female *Cx. tritaeniorhynchus*.

Japanese encephalitis (JE) virus is the most widespread and, in terms of human morbidity and mortality, the most important antigenically related mosquito-borne flavivirus which causes encephalitis in humans. The virus is found throughout much of eastern Asia, from the southeastern Soviet Union in the north to Indonesia in the south and India in the west; hence it occurs in temperate areas, where mosquitoes are not active in the winter, as well as in warmer areas. It is a mystery how the virus survives when its adult mosquito vectors are inactive or absent.

In studies showing that JE virus could be transmitted vertically by aedine mosquitoes,[1] we originally failed to demonstrate such transmission in *Culex tritaeniorhynchus*, the most important vector of the virus to humans and swine. Later, in a preliminary publication, we reported vertical transmission of JE virus to F1 larvae in this species.[2] We describe here further experimental work, carried out during 1977–1986, on vertical transmission of JE virus by *Cx. tritaeniorhynchus* and other species of mosquitoes.

MATERIALS AND METHODS

Mosquitoes

The origins of the *Cx. tritaeniorhynchus* strains employed are shown in Table 1. Experiments with the Nagasaki strain were carried out at 2 different sites (Baltimore and Taipei). Though the origin of the mosquitoes was the same, the colonies were obtained by the 2 laboratories from the Department of Medical Zoology, Nagasaki University School of Medicine, in 1976 and 1981, respectively, and hence had different passage histories. The *Cx. annulus* colony employed was started with material from Taiwan in 1964. The nomenclature of this species is open to question, and some consider that it should be called *Cx. vishnui*.[3] The geographic origin of the other colonies employed is indicated in the text or in Table 2. All field material was from Taiwan.

Viruses

Four strains of JE virus were employed. These were the Indonesia strain, isolated from a pool of *Cx. tritaeniorhynchus* collected near Jakarta, Indonesia in January 1979; the Osaka strain, isolated from a pool of *Cx. tritaeniorhynchus* collected near Osaka, Japan in August 1979; the Sagiyama strain, isolated from a pool of *Cx. tritaeniorhynchus* collected near Sagiyama, Japan in August 1956; and the Taiwan strain, isolated from a pool of *Cx. annulus* collected in Taiwan

TABLE 1

Geographic origin and date of colonization of Culex tritaeniorhynchus *strains employed*

Strain	Geographic origin	Year of colonization
Amami	Konia, Amami-Oshima Island, Japan	1972
Balloki	Balloki Headworks, Punjab, Pakistan	1968
Changa	Changa Manga National Forest, Punjab, Pakistan	1967
Japan	Japan (strain from U.S. Army 406th Medical General Laboratory)	1956
Nagasaki	Mogi-machi, Nagasaki, Japan	1965
re:e:ae	Laboratory hybrid of several strains	
Taiwan	Taipei, Taiwan	1971

in June 1972. None of the strains had ever been passed in cell cultures or mice, but all had been passed parenterally in *Toxorhynchites amboinensis*.

Viral assay

Most specimens to be tested for the presence of JE virus were assayed by inoculating triturates intrathoracically into *Tx. amboinensis* mosquitoes as described elsewhere.[4] Each mosquito received ~0.17 μl inoculum. *Tx. amboinensis* were held for 14 days at 28–32°C and then examined by the head-squash technique.[5] Five *Toxorhynchites* were usually examined for each sample; the test was not considered satisfactory, if negative, unless at least 3 were examined. The indirect fluorescent antibody technique was used with anti-JE mouse ascitic fluid and a conjugated anti-mouse goat serum. In a few instances, adult *Culex* were tested directly for JE virus by the head-squash technique.

Experimental design

Female mosquitoes, caged with males but not having had a bloodmeal, were infected with JE virus either by intrathoracic inoculation or by feeding on viremic chicks. The viremic chicks were infected 48–84 hr beforehand, when <24 hr old, by sc inoculation of virus. Preliminary studies with the Taiwan strain of virus and 10 chicks, each tested at 12 hr intervals, indicated that viremia could be expected to vary at $10^{6.0-8.0}$ ID_{50}/ml during this time interval, with a mean of $10^{6.5}$ ID_{50}/ml at 48 and 60 hr post-inoculation, and a mean of 10^{7} ID_{50} per ml at 72 and 84 hr post-inoculation.

Calculation of the ID_{50} was based on parenteral inoculation of *Tx. amboinensis*. Infection of parent females was confirmed at the completion of each experiment by examining head squashes of a sample of the survivors, at least 3 for experiments when females had been infected parenterally and at least 10 for experiments when

TABLE 2

Vertical transmission to F1 larvae of the Taiwan strain of Japanese encephalitis virus by various species of parenterally infected mosquitoes

Exp.	Mosquito species (geographic origin)	Days eggs laid (after infection)	No. examined	Pools pos exmained	MIR*
2110	*Cx. pipiens pallens* (Japan)	12–14	5,073	1/52	1:5,073†
2111	*Cx. pipiens molestus* (Japan)	12–13	3,556	3/3[illegible]	1:711
2124	*Cx. quinquefasciatus* (Indonesia)	12–15	7,800	[illegible]	1:2,600†
2127	*Cx. quinquefasciatus* (Vietnam)	12–15	6,400	[illegible]	1:6,400
2130	*Cx. quinquefasciatus* (Matsu Island	12–15	2,373	1/24	1:2,373
2141	*Cx. quinquefasciatus* (Taiwan; field)	12–16	9,354	7/94	1:1,336‡
2143	*Cx. quinquefasciatus* (Quemoy Island)	11–13	2,100	1/21	1:2,100‡
2112	*Armigeres subalbatus* (Taiwan; field)	15–18	869	14/36	1:62
2160	*Armigeres flavus* (Taiwan; field)	17–37	1,375	1/15	1:1,375§
2156-68	*Aedes alcasidi* (Taiwan)	12–18	655	2/7	1:328
1960	*Aedes vexans* (Hawaii)	9–20	2,334	1/24	1:2,334

* Minimum infection rate.
† Eight of 10 parent females positive by head squash.
‡ Nine of 10 parent females positive by head squash.
§ Five of 10 parent females positive by head squash.

females had been infected orally. Unless otherwise noted, all parent females examined were found positive.

After infection, parent females were maintained at 25–30°C. Mosquitoes, infected orally, were given the opportunity to oviposit as soon as their eggs were mature; those eggs were discarded. The interval of time between infection of parent females and the bloodmeal which yielded the F1 progeny examined varied from experiment to experiment. This, and the interval of time between the latter bloodmeal and oviposition, is specified in the Results. Except for infectious bloodmeals, mosquitoes were fed on laboratory mice.

Eggs from infected females were collected either as a group or separately by family, depending upon the experiment. Unless otherwise noted, F1 aquatic stages were reared and adults maintained at 25–30°C. In experiments in which progeny were reared by family, only families in which ≥20 progeny survived to be tested are included in the results. F1 progeny were examined in pools. Pool size was limited to 100 specimens and was often smaller. Both aquatic stages and adults, separated by sex, were triturated for assay in Ten Broeck tissue grinders in ~1.0 ml of PBS (pH 7.4) containing 30% heated calf serum (30 min at 56°C). After centrifugation at 4°C to remove debris, the undiluted supernatant fluid was tested for the presence of virus as described above.

RESULTS

Culex tritaeniorhynchus

We suspected that our original failure to detect JE virus in F1 adult *Cx. tritaeniorhynchus* was related to the temperature at which immature stages were reared, as demonstrated in vertical transmission of St. Louis encephalitis virus by aedine mosquitoes.[6] In order to select the most appropriate combination for more intensive study, several colonies of *Cx. tritaeniorhynchus* and 4 strains of JE virus were tested. In these experiments, parent females were infected parenterally and, since it soon became apparent that filial infection rates varied widely between individual females, progeny were examined by family.

Variation in vertical and filial infection rates among mosquito and virus strains

One set of experiments was carried out with 4 different colonies of *Cx. tritaeniorhynchus* in Baltimore; the results of these experiments are shown in Table 3. Parent females were allowed to feed on mice 5–7 days after they had been infected, and then allowed to begin ovipositing as soon as their eggs were mature (11–13 days after infection). F1 progeny were examined as fourth stage larvae. It will be noted that the vertical transmission rate (i.e., the percentage of females transmitting virus to 1 or more progeny) ranged from a high of 80%, in the case of the Balloki strain of *Cx. tritaeniorhynchus* infected with the Osaka strain of virus, to a low of 12%, for the re;e;ae strain of *Cx. tritaeniorhynchus* infected with the Indonesia stain of virus. In general, filial infection rates (i.e., the percentage of progeny infected) paralleled vertical transmission rates, with the Balloki strain infected with the Osaka virus having a minimum infection rate (MIR) of 1:32 and the re;e;ae strain infected with the Indonesia virus having a MIR of 1:360. While differences were not great, generally the Osaka strain gave the highest vertical and filial infection rates and the Sagiyama and Indonesia strains the lowest. In similar experiments carried out in Taiwan with the same strains of virus, the combination of the Osaka stain of virus with Nagasaki strain of mosquito appeared particularly promising (Table 4). In these experiments, parent females were allowed to feed on mice 7 days after they had been infected and were allowed to begin ovipositing 11 days after infection. The superiority of the combination of the Nagasaki strain of mosquito with the Osaka strain of virus was confirmed in further experiments in which parent females were infected orally (Table 5). In the later experiments, parent females were allowed to feed on mice 9 or 10 days after they had been infected, and allowed to begin ovipositing 12 or 13 days after infection.

Variation in filial infection rates among F1 families

Differences in filial infection rates among families were explored in detail in mosquitoes of the Nagasaki strain orally infected with the Osaka strain of virus by examining progeny in pools of 5 larvae. In 1 such experiment, among 6 families derived from eggs laid on the same day by females infected at the same time, 9 of 10 pools of 1 family were positive, as were 8 of 10 in another. However, 0 of 10 pools were positive in 2 other families, and 2 of 10 and 1 of 8

TABLE 3

Vertical transmission of 4 strains of Japanese encephalitis virus by 4 strains of parenterally infected Culex tritaeniorhynchus *mosquitoes*

Mosquito strain	Osaka virus				Sagiyama virus			
	F1 Families pos/examined (% pos)	F1 Individuals examined	F1 Pools pos/examined	MIR	F1 Families pos/examined (% pos)	F1 Individuals examined	F1 Pools pos/examined	MIR
Nagasaki	7/24 (29)	1,000	8/50	1:125	8/30 (27)	1,340	10/67	1:134
Changa	7/12 (58)	380	8/19	1:48	5/9 (56)	420	6/21	1:70
Balloki	20/25 (80)	1,260	39/63	1:32	2/8 (25)	400	2/20	1:200
re:e:ae	9/21 (43)	840	11/42	1:76	7/27 (26)	1,300	9/65	1:144
Totals	43/82 (52)	3,480	66/174	1:53	22/74 (30)	3,460	27/173	1:128
	Taiwan virus				Indonesia virus			
Nagasaki	28/36 (78)	2,340	48/117	1:49	14/29 (48)	1,680	30/84	1:56
Changa	15/37 (41)	1,820	25/91	1:73				
Balloki					6/20 (30)	1,400	7/70	1:200
re:e:ae					3/26 (12)	1,080	3/54	1:360
Totals	43/73 (59)	4,160	73/208	1:57	23/75 (31)	4,160	40/208	1:104

All F1 progeny were examined as larvae.

pools were positive in the other 2. Such variation was observed consistently and similar results were obtained when parent females were infected parenterally.

Effect of time of oviposition on vertical infection rate

In the course of the experiments with the different strains of *Cx. tritaeniorhynchus* and JE virus, it was noted that among eggs laid by a group of females infected at the same time, the last egg rafts laid were more apt to yield infected progeny than those laid earlier. At the time that such observations were first made, it was assumed that vertical transmission of JE virus was transovarial in nature; hence the data were puzzling. It was thought possible, for example, that those parent females more likely to transmit virus to their progeny had delayed oviposition because of an adverse effect of the virus.

To obtain further information on this phenomenon, further experiments were conducted. A large group of orally infected females was divided into lots of equal size immediately after the infective bloodmeal. All lots were allowed to begin ovipositing as soon as they were able to do so and those eggs were discarded. Subsequently, all lots were allowed to feed on a normal mouse on the same day. Thereafter, different lots were allowed to begin ovipositing at different times. As would be expected, not all egg rafts were laid

TABLE 4

Vertical transmission of 4 strains of Japanese encephalitis virus by various strains of parenterally infected Culex tritaeniorhynchus *mosquitoes*

Mosquito strain	Virus strain							
	Osaka		Taiwan		Sagiyama		Indonesia	
	F1 Families pos/examined (% pos)	No. exp.	F1 Families pos/examined (% pos)	No. exp.	F1 Families pos/examined (% pos)	No. exp.	F1 Families pos/examined (% pos)	No. exp.
Nagasaki	18/18 (100)	2	31/36 (86)	2	19/25 (76)	2		
Amami	52/91 (57)	5	26/52 (50)	4	24/50 (48)	3	13/41 (32)	3
Taiwan	9/18 (50)	1	22/56 (39)	3	3/15 (20)	1	2/8 (25)	1
Japan	11/34 (32)	1			13/36 (36)	1	7/36 (19)	1
Field	7/19 (37)	1	30/53 (57)	2	26/43 (60)	1	13/18 (72)	1
Totals	97/180 (54)		109/197 (55)		85/169 (50)		35/103 (34)	

All F1 progeny were exxmined as larvae.

TABLE 5

Vertical transmission of 3 strains of Japanese encephalitis virus by 2 strains of orally infected Culex tritaeniorhynchus *mosquitoes*

Mosquito strain	Virus strain: Osaka — F1 Families pos./examined (% pos)	Osaka — No. exp.	Taiwan — F1 Families pos./examined (% pos)	Taiwan — No. exp.	Sagiyama — F1 Families pos./examined (% pos)	Sagiyama — No. exp.
Nagasaki	122/150 (81)	11	31/49 (63)	2	33/70 (47)	3
Amami	10/74 (14)	4	10/25 (40)	2	14/28 (50)	1

All F1 progeny were examined as larvae.

on the first day that an oviposition receptacle was made available. The results of 2 such experiments are shown in Table 6. The phenomenon referred to earlier is clearly seen in the data for Lot 1 of Exp. 2426. Namely, eggs laid earlier yielded fewer infected progeny than those laid later. The data for Lot 2 in this experiment demonstrate that it is the time of oviposition that determines the infection rate and that the phenomenon cannot be explained by a proclivity of females more apt to transmit to oviposit later. The MIR for the 2,885 progeny of all females in Lot 1 was 1:244, whereas the MIR for the 2,949 progeny of all females in Lot 2 was 1:53, about 5 times higher. The data in Table 6 also demonstrates that the design of most of the vertical transmission experiments carried out to compare mosquito and virus strains was inappropriate. Since it was assumed in the beginning that vertical transmission was transovarial in nature, the bloodmeal intended to yield infected progeny was offered at the time that the overall virus content in the parent female was believed to be at a peak and the time of oviposition was ignored.

Transstadial transmission of virus to the adult stage

In studying vertical transmission of JE virus in *Cx. tritaeniorhynchus*, it was noted that the filial infection rate was almost always lower when measured in F1 adults as compared with F1 larvae. This is illustrated by the data in Table 7. These data were derived from experiments in which groups of F1 progeny were reared to the

TABLE 6

Vertical transmission of the Osaka strain of Japanese encephalitis virus by the Nagasaki strain of orally infected Culex tritaeniorhynchus *mosquitoes by time elapsed between infection and oviposition*

	Days eggs laid (after infection)	No. F1 families pos./examined (% pos)	Number F1 individuals examined	Number F1 pools pos. examined	MIR
		Experiment 2426			
Lot 1	13	0/12 (0)	829	0/43	<1:829
	14	1/10 (10)	805	1/42	1:805
	16	1/4 (25)	365	3/18	1:122
	17	3/7 (43)	523	5/28	1:105
	19	1/2 (50)	163	2/8	1:82
Lot 2	20	18/27 (67)	2,400	36/119	1:67
	21	4/4 (100)	269	11/13	1:24
	22	2/2 (100)	280	9/14	1:31
		Experiment 2433			
Lot 1	12	0/8 (0)	702	0/36	<1:702
	13	0/2 (0)	144	0/8	<1:144
Lot 2	15	1/9 (11)	722	1/38	1:722
	16	1/2 (50)	230	2/12	1:115
Lot 3	18	3/3 (100)	177	8/9	1:22
	19–22	4/5 (80)	451	13/23	1:35

All F1 progeny were examined as larvae.

TABLE 7

Vertical transmission to F1 larvae and adults of the Osaka strain of Japanese encephalitis virus by the Nagasaki strain of Culex tritaeniorhynchus *mosquitoes*

Exp.	Larvae			Adults		
	Individuals examined	Pools pos/ examined	MIR	Individuals examined	Pools pos/ examined	MIR
2349	780	39/39	1:20	883	17/*	1:52
2356	400	18/20	1:22	182	3/11	1:61
2365	305	15/15	1:20	480	6/19	1:80
2410	140	4/7	1:35	141	0/7	<1:141
2428	424	17/21	1:25	369	9/36	1:41
2487	40	1/1	1:40	39	1/*	1:39
2511	180	3/4	1:60	166	0/9	<1:166
2531	480	5/20	1:96	606	3/25	1:202
2539	488	16/26	1:31	540	2/26	1:270
2568	155	4/5	1:39	193	0/9	<1:193
2341F	220	11/11	1:20	120	0/12	<1:120
2350F	345	17/18	1:20	421	7/*	1:60
2426F	200	4/10	1:50	400	0/20	<1:400
2446F	241	2/16	1:121	305	1/32	1:305
2462F	1,071	17/56	1:63	690	1/96	1:690
2465F	280	10/15	1:28	320	7/*	1:46
2470F	553	11/29	1:50	679	2/43†	1:340
2474F	45	2/3	1:23	71	1/*	1:71
2479F	75	6/6	1:13	33	1/*	1:33
2494F	465	4/38	1:116	414	2/27	1:207
2513F	60	3/3	1:20	21	0/*	<1:21
2530F	600	6/30	1:100	1,287	0/54	<1:1,287
Totals	7,547	215/	1:35	8,360	63/	1:133

* Examined by squash.
† Examined partially by squash.
Parent females were infected orally in experiments with the suffix "F."

fourth larval stage and then randomly separated into 2 lots. One lot was assayed for virus at the end of the fourth larval stage and the other was allowed to develop to the adult stage before assay. Adult progeny were tested only if 1 or more pools of the corresponding larval lot was positive. Overall, the filial infection rate was about 4 times higher when measured by assay of F1 larvae as compared to F1 adults, but there was marked variation from experiment to experiment. No data were obtained which suggested that filial infection rates were different for the 2 sexes, or that the age of the F1 adults at the time of assay had an effect.

Attempts to determine if transstadial transmission of JE virus to the adult stage in *Cx. tritaeniorhynchus* is temperature dependent were frustrated by an inability to rear *Cx. tritaeniorhynchus* at low temperatures in the laboratory. All that could be accomplished was to rear mosquitoes at 25°C to the early fourth larval stage and then lower the temperature to 20°C for the remainder of the aquatic development period. Data obtained from 3 such experiments indicated that if the temperature at which larvae were reared had an effect, it was in the opposite direction from that anticipated. In these experiments, which were carried out with parenterally infected females of the Taiwan and Japan strains of *Cx. tritaeniorhynchus* and the Taiwan or Osaka strains of virus, 23 positive pools were found among a total of 13,138 adult progeny from larvae reared entirely at 25°C (MIR = 1:571), as compared with only 1 positive pool among a total of 7,108 adult progeny from larvae of the same lots reared in part at 20°C (MIR = 1:7,108).

Culex annulus

While relatively little work was carried out with *Cx. annulus* as compared to *Cx. tritaeniorhynchus*, the data that were obtained were similar. The data from 1 experiment with *Cx. annulus* infected with the Osaka strain of virus are

TABLE 8

Vertical transmission to F1 larvae and adults of the Osaka strain of Japanese encephalitis virus by a laboratory strain of orally infected Culex annulus *mosquitoes*

Days eggs laid after infection	No. F1 larvae examined	No. F1 larval pools pos. examined	MIR	No. F1 adults examined	No. F1 adult pools pos examined	MIR
14	2,361	0/24	< 1:2,361	2,432	0/25	< 1:2,432
18	809	5/8	1:162	727	1/8	1:727
19	888	8/9	1:111	494	1/5	1:494
20	331	1/4	1:331	95	0/2	< 1:95

shown in Table 8. At the conclusion of this particular experiment, only 13 of 17 (78%) of the surviving parent females were found positive. Consequently, the filial infection rates for progeny derived from infected females was probably higher than those shown in Table 8. It will be noted that, as for *Cx. tritaeniorhynchus*, the time of oviposition affected the filial infection rate and the rate for F1 adults was lower than for F1 larvae.

In another experiment, wild-caught *Cx. annulus* parent females were parenterally infected with the Taiwan strain of virus; 10 of 18 F1 families were found positive when tested in the larval stage. There were 23 positive pools among a total of 2,198 progeny tested (MIR = 1:96). The eggs from which positive progeny were derived were laid 18–19 days after parental infection.

Other mosquito species

Data on vertical transmission of JE virus by various other mosquito species that could be involved in the ecology of JE virus in nature are shown in Table 2. It will be noted that, with the exception of *Armigeres subalbatus*, filial infection rates were low as compared with those observed for *Cx. tritaeniorhynchus* and *Cx. annulus*. Most of the experiments were carried out before the importance of the time of oviposition was known; hence, the rates are probably lower than would be observed under optimum conditions.

While the data in Table 2 refers to F1 progeny tested in the larval state, F1 adult progeny of *Cx. quinquefasciatus* (Vietnam strain) were found positive in other experiments. Two positive pools were found among 8,334 adult progeny (MIR = 1:3,167) of females parenterally infected with the Taiwan strain of virus. Similarly, a high proportion of F1 adult progeny of *Ar. subalbatus* were consistently found positive in experiments not included in Table 2.

Virus infection and duration of larval development

It was noted consistently in vertical transmission experiments with both *Culex* and aedine species that, among F1 progeny which hatch at the same time, those that pupate late are more apt to be infected than those which pupate early. This could be because viral infection affects the rate of larval development, or because the duration of larval development affects viral expression.

The following experiment was carried out to determine if the latter explanation might be correct. About 4,000 eggs from *Aedes albopictus* parent females (Oahu strain) parenterally infected with the Taiwan strain of JE virus were hatched within 4.5 hours. Half of the larvae were placed in 10 rearing pans (36 cm long, 23 cm wide, 5 cm deep) at a density of ~200 larvae/pan; the other 2,000 larvae were placed in a single pan of the same size. Initially, each pan was furnished the same amount of food. Ten days after hatching, the larvae which had been crowded in the single pan were redistributed in 10 pans and fed optimally thereafter. The result of the above manipulations was that the larvae which had initially been distributed optimally pupated 7–10 days (median = 8 days) after hatching, whereas those that were crowded initially pupated 7–17 days (median = 16 days) after hatching. All the F1 progeny were examined for infection as adults 4 days after eclosion. Three positive pools were found among 1,660 adults derived from the larvae reared in an optimum manner initially, and 3 positive pools were found among the 1,537 adults derived from the larvae initially reared under crowded conditions.

Sexual transmission of JE virus by male Cx. tritaeniorhynchus

Inconsistent results were obtained in several attempts to determine if male *Cx. tritaeniorhynchus* mosquitoes infected with JE virus could transmit the virus sexually to females. Sometimes no infection could be detected in the females, despite the presence of spermatozoa in their spermathecae, and at other times the females did become infected. In 1 experiment in which positive results were obtained, 14 females of the Nagasaki strain were examined 14 days after they had been caged with males which had been infected parenterally 7 days previously with the Sagiyama strain of JE virus. At the time of examination, the spermathecae of 6 of the females contained spermatozoa and those of the 8 others did not. All 6 of the former females were found infected with JE virus, whereas only 3 of the latter were positive.

DISCUSSION

It seems clear that vertical transmission of JE virus by *Cx. tritaeniorhynchus* mosquitoes is affected by the genetic composition of both the mosquito and the virus. Though relatively few mosquito and virus strains were employed in our experiments, and most of the mosquitoes had been colonized for some time, the results of the experiments with field-collected mosquitoes (Table 4) suggest that the results obtained in the laboratory are not very different from what occurs in nature.

Variation in the vertical transmission rate (the percentage of parent females transmitting) among the various *Cx. tritaeniorhynchus* mosquito-virus combinations was less marked than that of the filial infection rate (the percentage of progeny infected), even when the latter was measured in families of the same mosquito-virus combination. In general, with this mosquito species and as measured by assay of larvae, one could expect vertical transmission rates of 20–80% and filial infection rates among individual families of up to 20%.

It appears that vertical transmission of JE virus in *Cx. tritaeniorhynchus* is not transovarial in nature. Rather, the egg is infected at the time of oviposition, as has been reported for JE virus in *Aedes* mosquitoes.[8]

The explanation for the relatively inefficient transstadial transmission of JE virus to the adult stage in *Cx. tritaeniorhynchus* (at least in the laboratory) is not apparent. In so far as it could be tested, temperature did not appear to be the explanation. It should be noted, however, that while we were unable to rear this species successfully in the laboratory at low temperatures, we have observed healthy larvae in the field in water as cold as 13°C.

Though relatively few experiments were carried out with *Cx. annulus*, vertical transmission of JE virus in this species seemed to parallel that in *Cx. tritaeniorhynchus*. Experimental vertical transmission of JE virus in the same or a closely related species (*Cx. vishnui*) has been reported previously in India.[9] Unfortunately, the nature of vertical transmission of JE virus in mosquitoes (i.e., infection at the time of oviposition) was not discovered until all of the experiments with mosquito species other than *Cx. tritaeniorhynchus* had been completed. Nevertheless, the comparative results are probably valid since early experiments with all species were carried out in a comparable manner. While vertical transmission of JE virus was demonstrated with *Cx. pipiens* and *Cx. quinquefasciatus*, filial infection rates were lower than for *Cx. tritaeniorhynchus* and *Cx. annulus* corresponding to the relative susceptibility of the various species to oral infection with JE virus (data not shown). On the other hand, high filial infection rates were observed consistently with *Armigeres subalbatus* mosquitoes, though we were unable to infect this species orally by feeding on viremic chicks (data not shown).

As shown by the experiment in which larval development was deliberately retarded, it is not the length of the larval period that is responsible for the higher filial infection rates noted among late pupating larvae. This suggests that the viral infection itself could retard larval development. It is also possible that another factor may be responsible both for a longer larval development period and increased susceptibility to vertically transmitted viral infection.

There can be little doubt that vertical transmission of JE virus occurs in *Cx. tritaeniorhynchus* mosquitoes in nature. The virus has, in fact, been recovered from larvae collected in the field[10] and from male mosquitoes reared from such larvae (V. Dhanda, National Institute of Virology, Pune, India, personal communication). However, the question of whether vertical transmission of JE virus in this species is the

mechanism by which the virus survives winter and other adverse periods can only by answered by field studies in which both the frequency and the timing of such transmission is taken into account. The vertical transmission rates demonstrated in this study are relatively low compared to those reported for certain bunyaviruses; however, these data should be examined in relation to other factors, such as the size of the mosquito population in question. In view of the enormous size of the *Cx. tritaeniorhynchus* populations in many endemic areas, it would not take a very high rate of vertical transmission to assure the survival of the virus from one season to another.

Cx. tritaeniorhynchus may be responsible for inter-seasonal survival of the virus in some areas. However, different mosquito species or other mechanisms must be involved in others, as *Cx. tritaeniorhynchus* does not occur everywhere that the virus is present. Mosquito species which may be important in the transmission of the virus to humans or domestic animals may not be those essential to the survival of the virus. Relatively little is known of the susceptibility to JE virus of mosquito species that do not feed on humans or domestic animals, or of the JE viremia levels that might be encountered by mosquitoes in nature, especially in areas unaltered by human activity. Moreover, there is no apparent reason why a species relatively resistant to oral infection could not be important in the ecology of a virus if its relative lack of susceptibility were compensated by the size of its population.

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Vertical transmission of Japanese encephalitis virus to the F1 adult stage was demonstrated in Culex tritaeniorhynchus, Cx. annuals, Cx. quinquefasciatus, and Armigeres subalbatus. Transmission to the F1 larval stage was demonstrated in Cx. pipiens, Aedes vexans, Ae. alacasidi, and A. flavus. In Cx. tritaeniorhynchus, vertical transmission rates (the percentage of parent females transmitting to progeny) varied (12-100%). Filial infection rates (the percentage of progeny infected) for a given mosquito virus combination were markedly affected by the interval of time between parental infection and oviposition, suggesting that vertical infection was not transovirial in nature but occured at oviposition. Filial infection rates for Cx. tritaeniorhynchus also varied widely by family and, as measured in F1 larvae, rates in excess of 20% were observed in a family. Filial infection rates in Cx. tritaeniorhynchus F1 adults were about 4 times lower than those in larvae. Japanese encephalitis virus was sexually transmitted from male to female Cx. tritaeniorhynchus.

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